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# Heterocomplex formation of 5-$HT_{2A}$-$mGlu_2$ and its relevance for cellular signaling cascades

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ABSTRACT

Dopamine, serotonin and glutamate play a role in the pathophysiology of schizophrenia. In the brain a functional crosstalk between the serotonin receptor 5-$HT_{2A}$ and the metabotropic glutamate receptor $mGlu_2$ has been demonstrated. Such a crosstalk may be mediated indirectly through neuronal networks or directly by receptor oligomerization. A direct link of the 5-$HT_{2A}$-$mGlu_2$ heterocomplex formation to receptor function, i.e. to intracellular signaling, has not been fully demonstrated yet. Here we confirm the formation of 5-$HT_{2A}$-$mGlu_2$ heterocomplexes using quantitative Snap/Clip-tag based HTRF methods. Additionally, $mGlu_2$ formed complexes with 5-$HT_{2B}$ and $mGlu_5$ but not 5-$HT_{2C}$ indicating that complex formation is not specific to the 5-$HT_{2A}$-$mGlu_2$ pair. We studied the functional consequences of the 5-$HT_{2A}$-$mGlu_2$ heterocomplex addressing cellular signaling pathways. Co-expression of receptors in HEK-293 cells had no relevant effects on signaling mediated by the individual receptors when $mGlu_2$ agonists, antagonists and PAMs, or 5-$HT_{2A}$ hallucinogenic and non-hallucinogenic agonists and antagonists were used. Hallucinogenic 5-$HT_{2A}$ agonists induced signaling through $G_{q/11}$, but not $G_i$ and thus did not lead to modulation of intracellular cAMP levels. In membranes of the medial prefrontal cortex [$^3$H]-LY341495 binding competition of $mGlu_{2/3}$ agonist LY354740 was not influenced by 2,5-dimethoxy-4-iodoamphetamine (DOI). Taken together, the formation of GPCR heterocomplexes does not necessarily translate into second messenger effects. These results do not put into question the well-documented functional cross-talk of the two receptors in the brain, but do challenge the biological relevance of the 5-$HT_{2A}$-$mGlu_2$ heterocomplex.



## 1. Introduction

Schizophrenia is a devastating mental disorder that affects about 1% of the population over the lifetime (Freedman, 2003; Sawa and Snyder, 2003; Tamminga and Holcomb, 2005). While the etiology of the disease is not completely understood, three neurotransmitter systems seem to be particularly relevant: dopamine, serotonin and glutamate. Currently marketed antipsychotic drugs block the dopamine $D_2$ receptor at therapeutic exposures (Carlsson, 1978; Talbot and Laruelle, 2002). Several studies demonstrated psychotomimetic effects of hallucinogenic compounds, like LSD, mescaline and DOI, due to 5-$HT_{2A}$ receptor agonism (Geyer and Vollenweider, 2008; Gonzalez-Maeso et al., 2007; Schreiber et al., 1994), which were attenuated by selective 5-$HT_{2A}$ receptor antagonists (Vollenweider et al., 1998), and by atypical antipsychotics (Meltzer et al., 1989). Preclinical and clinical studies found that channel blocking *N*-methyl-D-aspartate (NMDA) receptor antagonists (e.g., ketamine and phencyclidine) induce a syndrome in healthy volunteers resembling positive and negative symptoms of schizophrenia, suggesting that modulation of the glutamate transmitter system could be a valid approach to treat schizophrenia. Indeed, a metabotropic glutamate receptor ($mGlu_{2/3}$) agonist was found to be efficacious during a phase II clinical trial in schizophrenia (Patil et al., 2007). In this study LY2140023, a prodrug of the $mGlu_{2/3}$ receptor orthosteric agonist LY404039, attenuated both positive and negative symptoms to a degree comparable to the atypical antipsychotic olanzapine. Together with results from subsequent clinical trials (Kinon et al., 2011; Stauffer et al., 2011)

*Abbreviations:* 5-HT, 5-hydroxytryptamine; 5-$HT_{2A}$, serotonin receptor 2A; DOI, 2,5-dimethoxy-4-iodoamphetamine; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; HTRF, homogenous time-resolved FRET; mGlu, metabotropic glutamate receptor; mPFC, medial prefrontal cortex; PAM, positive allosteric modulator; Tet, tetracycline.

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these data indicate the relevance of $mGlu_{2/3}$ receptor agonists as a novel antipsychotic principle, which had been suggested based on preclinical cytochemical, neurochemical and behavioral studies (Mezler et al., 2010; Moghaddam and Adams, 1998).

Besides direct activation of $mGlu_{2/3}$ receptors, such orthosteric agonists also modulate the activity of the 5-$HT_{2A}$ serotonin receptor subtype. A reciprocal functional inhibition of 5-$HT_{2A}$ agonism and $mGlu_{2/3}$ agonism has been described in the prefrontal cortex of rat (Aghajanian and Marek, 1999; Marek et al., 2000). In these studies, 5-$HT_{2A}$ receptor activation induced excitatory postsynaptic currents (EPSCs) in the medial prefrontal cortex (mPFC) (Aghajanian and Marek, 1999), and a $mGlu_{2/3}$ antagonist further enhanced the frequency and amplitude of EPSCs (Marek et al., 2000). By contrast $mGlu_{2/3}$ agonists or 5-$HT_{2A}$ receptor antagonists suppressed EPSCs and attenuated behavioral effects of serotonergic hallucinogens (*e.g.* LSD) (Gewirtz and Marek, 2000) and dissociative anesthetics (*e.g.* PCP) (Moghaddam and Adams, 1998). These findings are consistent with a proposed inhibitory autoreceptor function of $mGlu_{2/3}$ and suggest that presynaptic glutamate release is positively regulated by 5-$HT_{2A}$ receptor activation. In particular these results demonstrate that the effects of $mGlu_2$ and 5-$HT_{2A}$ receptor modulation are functionally antagonistic in the intact brain.

While the efficacy of the known $mGlu_{2/3}$ cross-reactive orthosteric agonists can not unequivocally be attributed to either of the two receptor types, novel $mGlu_2$-selective positive allosteric modulators (PAMs) like BINA generated similar effects (Benneyworth et al., 2007). Together with studies in $mGlu_2$ and $mGlu_3$ knockout mice (Spooren et al., 2000; Woolley et al., 2008), these data support the idea that $mGlu_2$, but not $mGlu_3$ is responsible for the functional 5-$HT_{2A}$ antagonism.

Since functional antagonism between 5-$HT_{2A}$ and $mGlu_2$ receptors is well established, it was of particular interest to investigate a possible direct molecular interaction between these two receptors. 5-$HT_{2A}$ and $mGlu_2$ receptors are both localized in brain cortex (Marek et al., 2000), but direct demonstration of the existence of 5-$HT_{2A}$-$mGlu_2$ heterocomplexes was made only recently by Gonzalez-Maeso et al. (2008). This study found that $mGlu_2$ and 5-$HT_{2A}$ directly interact in recombinant cell lines and are present in the same neuronal cells in culture. Additionally, these findings implicate functional consequences related to the pharmacology of antipsychotics due to interactions at the 5-$HT_{2A}$-$mGlu_2$ complex. It was shown that the formation of the 5-$HT_{2A}$-$mGlu_2$ complex enhances $G_{\alpha i}$ activation of hallucinogenic 5-$HT_{2A}$ agonists, which is described to be involved in hallucinogen-specific signaling (Gonzalez-Maeso et al., 2008, 2007). Activation of the $mGlu_2$ component suppresses the neuropsychological effects of hallucinogens. A further study reported that a 5-$HT_{2A}$-$mGlu_2$ heterocomplex serves as integration point balancing $G_{\alpha i}$- and $G_{\alpha q}$-dependent signaling (Fribourg et al., 2011). This indicates a direct crosstalk between the two receptors and remodels the idea of a synaptic mechanism of 5-$HT_{2A}$-induced glutamatergic transmission.

To further substantiate these findings, we co-expressed $mGlu_2$ in an inducible manner within a constitutive 5-$HT_{2A}$ background in HEK-293 cells. We determined the reciprocal influence of the two receptor types on receptor expression. Also, we evaluated the pharmacological responses of 5-$HT_{2A}$ and $mGlu_2$ by measuring intracellular calcium and cAMP levels upon stimulation with agonists, antagonists, hallucinogens and PAMs. We confirm the 5-$HT_{2A}$-$mGlu_2$ heterocomplex formation, and demonstrate that $mGlu_2$ additionally interacts with 5-$HT_{2B}$ and $mGlu_5$, but not with 5-$HT_{2C}$. The heterocomplex formation of 5-$HT_{2A}$-$mGlu_2$ alone did not result in a functional crosstalk. Furthermore, an effect of hallucinogenic 5-$HT_{2A}$ agonists on $G_{\alpha i}$-mediated signaling could not be observed. While our data do not question the functional crosstalk of these neurotransmitter systems in the brain, these results argue that the functional interaction of $mGlu_2$ and 5-$HT_{2A}$ may not be mediated by interaction in form of heteromers.

## 2. Materials and methods

### 2.1. Materials

LANCE cAMP 384 kit was purchased from PerkinElmer (Rodgau, Germany), FLIPR calcium 4 assay kit from Molecular Devices (Ismaning, Germany), and TagLite HTRF reagents were purchased from Cisbio Bioassays (Codolet, France). Compounds LY341495, LY354740, LY379268, LY404039, LY487379, BINA, 5-HT, 2-methylserotonin, R-(+)lisuride, Ketanserin, Mianserin, DOI (2,5-dimethoxy-4-iodoamphetamine), Mescaline, and [$^3$H]LY341495 can be obtained commercially (Tocris, Bristol, UK; Merck, Darmstadt, Germany; Sigma–Aldrich, Munich, Germany; and Selleck Chemicals, Houston, TX, USA). MGS0039 was synthesized at Abbott. All other reagents were purchased from Sigma–Aldrich.

### 2.2. Generation of cDNA constructs and cell lines

Plasmids encoding 5-$HT_2$ receptor subtypes A-C or $mGlu_5$ N-terminally fused to the Snap tag (pSnap-HTR2A, pSnap-HTR2B, pSnap-HTR2C, and pSnap-hGRM5) and $mGlu_2$ N-terminally fused to the Clip tag (pClip-hGRM2) were purchased from Cisbio Bioassays.

T-REx-293 cells (Invitrogen, Darmstadt, Germany) were cultured in DMEM (Invitrogen) with 5 μg/mL blasticidin S HCl (Invitrogen) and selected with 800 μg/mL geneticin (Invitrogen) for stable 5-$HT_{2A}$ (pcDNA3.1; Invitrogen) expression and with 150 μg/mL hygromycin B (Roche, Mannheim, Germany) for tetracycline-inducible $mGlu_2$ (pcDNA5/TO) expression (293-$H_{2A}$-i$G_2$). Additionally, cell lines expressing solely inducible $mGlu_2$ (293-i$G_2$) or 5-$HT_{2A}$ (293-$H_{2A}$) were generated. Maximal $mGlu_2$ expression was induced by 48 h stimulation with 1 μM tetracycline. For protein interaction assays, HEK 293T/17 cells (ATCC #CRL-11268) were transiently transfected using Lipofectamine (Invitrogen). 293T/17 cells showed better transfection rates and higher expression levels than regular HEK-293 cells and were thus used for all transient expression experiments. All cells were cultured at 37 °C under 5% $CO_2$ atmosphere in DMEM high glucose GlutaMAX-I (Invitrogen), 10% charcoal/dextran treated FBS (Thermo Scientific HyClone, Bonn, Germany), gentamycin (Invitrogen), and appropriate selection antibiotics.

### 2.3. HTRF assay

To measure HTR-FRET signals, 293T/17 cells were co-transfected with Snap-tagged 5-$HT_{2A}$, 5-$HT_{2B}$, 5-$HT_{2C}$, or $mGlu_5$ receptor and Clip-tagged $mGlu_2$ receptor 24 or 48 h prior to assay performance. Cells were labeled with Tag-lite Clip-Lumi4-Tb, Snap-Lumi4-Tb, and/or Clip-Red according to the manufacturer's protocol (Cisbio Bioassays) and HTR-FRET signal (665 nm) and Lumi4-Tb donor signal (620 nm) were measured using a PheraStar Plus (BMG Labtech, Ortenberg, Germany). HTRF ratio (665 nm/620 nm $\times$ $10^4$) was calculated to eliminate quenching and dispensing errors. Co-transfections and substrate labeling were carefully titrated to obtain expression and labeling ratios of 1.

### 2.4. cAMP assay

Determination of intracellular cAMP through time resolved fluorescence energy transfer (TR-FRET) was studied with the LANCE cAMP detection kit according to the manufacturer's instructions using 96-well half area microplates (Costar, Corning, Amsterdam, The Netherlands). Cells were serum-starved for five hours and stimulation with compounds was done for 30 min. For $mGlu_2$ receptor antagonist and PAM testing, cells were simultaneously stimulated with $EC_{80}$ or $EC_{20}$ concentrations of glutamate, respectively. Pre-stimulation of $mGlu_2$ with LY404039 ($EC_{80}$) or LY341495 ($EC_{100}$) was done for 5 h prior to 5-$HT_{2A}$ receptor stimulation with DOI.

### 2.5. $Ca^{2+}$ mobilization assay

Calcium mobilization was measured by FLIPR (Fluorometric Imaging Plate Reader; Molecular Devices) with the calcium-specific indicator assay calcium 4 according to manufacturer's protocol. Stable T-REx-293 cell lines were seeded in 96-well ($4 \times 10^4$ cells/well) clear-bottomed poly-D-lysine coated black microplates (Greiner Bio-One, Frickenhausen, Germany) two days prior to assay performance and incubated in glutamate and serum free medium for 5 h before the measurements.

### 2.6. Radioligand binding assay

Preparation of membranes and binding assay were performed as described by Wright et al. (2001). Assay volume of 500 μl consisted of 1 nM [$^3$H]LY341495, 14 μg protein of rat cerebral cortex membranes (Wistar Wistar) in 10 mM potassium phosphate and 100 mM potassium bromide, pH 7.6. Non-specific binding was

defined with 1 mM L-glutamate. Assay samples were incubated on ice for 30 min. Reactions were terminated by filtering through polyethyleneimine-presoaked Whatman GF/B glass fiber filters followed by rinsing with 3 mL ice-cold buffer for 10 s. 4 mL of Ultima Gold™ XR (Packard) was added to each filter and radioactivity was measured by liquid scintillation counting (Packard Tricarb model 2000 or 2200 CA). $K_i$ values were determined by nonlinear regression analysis of the original data (duplicate or triplicate determinations). Fitting was performed according to formulae described (Feldman, 1972; Munson and Rodbard, 1980).

### 2.7. Data analysis

Functional data were analyzed using Microsoft Excel and GraphPad Prism 5.03 software. Experiments were performed 3–6 times in triplicates. Nonlinear regressions and $EC_{50}$ determinations were done using the four parameter model. For statistical analysis data were first tested for Gaussian distribution and equal variances. Statistical differences of $EC_{50}$ values were determined using unpaired two-tailed *t*-test or Mann Whitney test. Significance of HTRF signals was tested by one-way analysis of variance (Kruskal–Wallis) and Dunn's post test. A *p*-value of $<0.05$ was considered as statistically significant.

## 3. Results

Previous studies have demonstrated a functional crosstalk of glutamatergic and serotonergic transmitter systems (Aghajanian and Marek, 1999; Gewirtz and Marek, 2000; Marek et al., 2000). Reciprocal influence of receptor binding and signaling has been described recently (Fribourg et al., 2011; Gonzalez-Maeso et al., 2008), suggesting that the direct interaction of $mGlu_2$ and 5-$HT_{2A}$ mediates the functional crosstalk. As the scope of the direct interaction studies was limited with respect to the elucidation of pharmacological relevance and selectivity of the interactions, we intended to further substantiate these findings by investigating the relevance of the physical receptor interaction for cellular signaling.

### 3.1. $mGlu_2$ and 5-$HT_{2A}$ receptors are in close proximity

First we wanted to confirm the described interaction of the $mGlu_2$ with the 5-$HT_{2A}$ receptor (Gonzalez-Maeso et al., 2008). In order to explore the selectivity of the interaction other 5-$HT_2$ receptors (2B and 2C) and $mGlu_5$ were tested for heterocomplex formation with the $mGlu_2$ receptor. A HTRF (homogenous time-resolved FRET) assay was employed to study a potential receptor interaction, as initial experiments using a classical FRET assay in cells co-expressing $mGlu_2$-GFP and RFP-tagged 5-HT receptors led to variable results (data not shown). The HTRF assay is based on receptors fused to Snap or Clip tags, respectively, which can be specifically labeled with HTRF fluorophores. The test is better suited to study the interaction of cell surface proteins, as only receptors localized at the cell surface are labeled (Supplementary Fig. 1), excluding non-specific labeling of the cells due to potential intracellular receptor localization. Further advantages of this assay are elimination of background and 'bleed-through' signals as well as the possible quantitative analysis of occurring HTRF (Doumazane et al., 2010; Maurel et al., 2008).

Snap- and Clip-tagged receptors were expressed in 293T/17 cells. Since we intended to compare the HTRF signals obtained with different receptor pairs, comparable expression levels of the respective two receptors (ratio of 1) were crucial for our assay. Surprisingly, expression levels of the different receptor subtypes varied substantially when the cells were transfected with equal DNA amounts (Supplementary Fig. 2A). In addition receptor expression levels were reciprocally influenced upon co-transfection. Therefore we titrated DNA amounts in cells co-transfected with Snap- and Clip-tagged receptor constructs and separately labeled the cells with Snap-Lumi4-Tb or Clip-Lumi4-Tb, respectively (Supplementary Fig. 2B). With this method the optimal DNA ratios to achieve comparable receptor expression levels were defined (Supplementary Table 1).

HTRF assays conducted under optimized conditions (equivalent expression levels are shown in Fig. 1B) revealed specific HTRF signals upon co-expression of Snap-5-$HT_{2A}$ with Clip-$mGlu_2$ indicating close proximity and thus an interaction of the receptors (Fig. 1A). Under the same conditions $mGlu_2$ and the 5-$HT_{2C}$ receptor subtype did not reveal HTRF signals. Similar results have been described by Gonzalez-Maeso et al. (2008). Additionally, specific HTRF signals could be obtained from 5-$HT_{2B}$-$mGlu_2$ and $mGlu_2$-$mGlu_5$ receptor pairs (Fig. 1), indicating potential heterocomplex formation of $mGlu_2$ with 5-$HT_{2A}$, 5-$HT_{2B}$ and $mGlu_5$ but not 5-$HT_{2C}$. Control experiments performed by separate incubation of co-transfected cells with Snap or Clip substrates (Fig. 1A), and by HTRF measurement of cells expressing either Snap or Clip constructs (Supplementary Fig. 3) did not show HTRF signals.

### 3.2. Functional interaction of $mGlu_2$ and 5-$HT_{2A}$ receptors

So far, a limited dataset is available describing how the pharmacology of the receptors is influenced by the formation of a GPCR heterocomplex in mammalian cells (Fribourg et al., 2011; Gonzalez-Maeso et al., 2008). To study the functional effects of $mGlu_2$-5-$HT_{2A}$

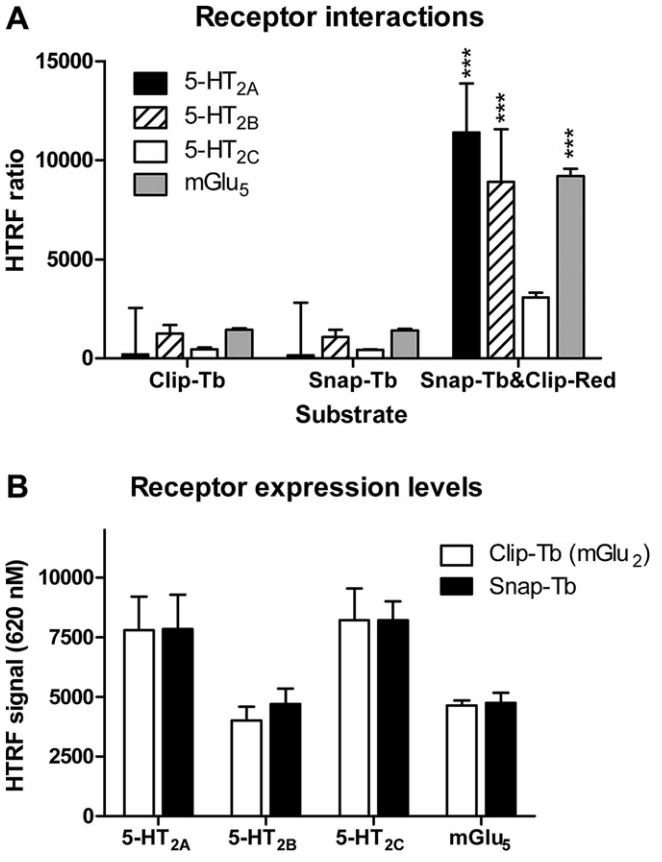


**Fig. 1.** 5-$HT_{2A}$, 5-$HT_{2B}$, and $mGlu_5$ interact with $mGlu_2$. 293T/17 cells were co-transfected with Clip-$mGlu_2$ and Snap-5-$HT_{2A}$, Snap-5-$HT_{2B}$, Snap-5-$HT_{2C}$, or Snap-$mGlu_5$. Cells were labeled by separate incubation with Snap-Lumi4-Tb or Clip-Lumi4-Tb to determine expression levels (B) or co-labeled with Snap-Lumi4-Tb and Clip-Red to detect HTRF signals (A). Fluorescence was measured at 620 nm (Lumi4-Tb) and 665 nm (Clip-Red, HTRF). High HTRF ratios (665 nm/620 nm × 10,000) intensities indicate protein interactions. Signals were specific since no bleed-through was observed in single labeled cells (A). Co-expression of Clip-$mGlu_2$ with Snap-5-$HT_{2A}$, -5-$HT_{2B}$, or -$mGlu_5$, but not with Snap-5-$HT_{2C}$, resulted in high HTRF signal ratios indicating specific interaction of these two receptors. Error bars indicate SD. Differences were tested for statistical significance by one-way ANOVA (Kruskal–Wallis) and Dunn's post test (*** $p \leq 0.001$; $n = 3$–7).

heterocomplex formation on the pharmacological responses of both receptors, several functional assays were performed.

Recombinant HEK-293 cell lines stably expressing tetracycline-inducible $mGlu_2$ receptor on a wild type and on a constitutive 5-$HT_{2A}$ expression background were generated (293-$H_{2A}$, 293-$iG_2$, and 293-$H_{2A}$-$iG_2$). Stimulation with tetracycline resulted in $mGlu_2$ mRNA upregulation in the inducible cell lines (Supplementary Fig. 4).

$mGlu_2$ is negatively coupled to cAMP formation via $G_{\alpha i}$ proteins (Conn and Pin, 1997). Thus, the influence of 5-$HT_{2A}$ co-expression on $mGlu_2$ receptor signaling was determined by measuring the $G_{\alpha i}$-mediated decrease of forskolin-induced cAMP levels. 293-$iG_2$ and 293-$H_{2A}$-$iG_2$ cells were stimulated with the $mGlu_{2/3}$ agonists LY354740, LY379268, and LY404039. There were no relevant differences of agonist potencies or efficacies between the two cell lines (Fig. 2A), indicating that the complex formation with 5-$HT_{2A}$ has little or no influence on $mGlu_2$ cAMP signaling. The rank order of $mGlu_2$ receptor agonist potencies was LY379268 > LY354740 > LY404039 >> glutamate (see Table 1). There was only a minor reduction of the $EC_{50}$ of LY354740 upon 5-$HT_{2A}$ co-expression ($EC_{50}$ 7.6 nM in 293-$iG_2$ and 3.5 nM in 293-$H_{2A}$-$iG_2$ cells; $p < 0.01$). Additionally, the effects of 5-$HT_{2A}$ co-expression on $mGlu_2$ receptor antagonists MGS0039 and LY341495 were tested after pre-stimulation with $EC_{80}$ concentrations of glutamate (25 μM). Co-expression of 5-$HT_{2A}$ did not significantly change the potency of either antagonist (Fig. 2C, Table 1).

A slight leftward shift of the selective $mGlu_2$ receptor PAM (positive allosteric modulator) concentration-response-curves in 293-$H_{2A}$-$iG_2$ cells compared to 293-$iG_2$ cells was observed, resulting in a minor enhancement of $mGlu_2$ PAM potencies (Fig. 2B and Table 1). This effect was more prominent for the PAM LY487379 ($EC_{50}$ 221.9 nM in 293-$iG_2$ cells and 83.7 nM in 293-$H_{2A}$-$iG_2$ cells) than for BINA ($EC_{50}$ 24.1 nM in 293-$iG_2$ cells and 14.1 nM in 293-$H_{2A}$-$iG_2$ cells). This finding is surprising, as the interaction of $mGlu_2$ with 5-$HT_{2A}$ was described to be antagonistic (Gonzalez-Maeso et al., 2008; Marek et al., 2000). If there is a functional effect of $mGlu_2$-5-$HT_{2A}$ heterocomplex formation, our data indicate a rather enhancing influence of 5-$HT_{2A}$ co-expression on the allosteric modulation of the $mGlu_2$ receptor.

For investigation of the influence of $mGlu_2$ co-expression on $G_{\alpha q}$-mediated 5-$HT_{2A}$ receptor signaling the intracellular $Ca^{2+}$ mobilization induced by 5-$HT_{2A}$ receptor stimulation was assessed. The response to non-hallucinogenic 5-$HT_{2A}$ agonists was not affected by $mGlu_2$ receptor co-expression (Fig. 3A). Potencies and efficacies of 5-HT, 2-methylserotonin (2-MS), and R-(+)lisuride measured in 293-$H_{2A}$-$iG_2$ cells did not differ from 293-$H_{2A}$ cells (Table 2). Since differential responses of 5-$HT_{2A}$ stimulation by hallucinogenic and non-hallucinogenic compounds have been reported (Cussac et al., 2008; Gonzalez-Maeso et al., 2007; Schmid and Bohn, 2010) we also tested the effect of mescaline and DOI. Potency and efficacy of the hallucinogenic compounds were likewise not changed by co-expression of $mGlu_2$ (Fig. 3A, Table 2). In our hands, all 5-$HT_{2A}$ agonists induced a full activation of the receptor. Also, the response to the 5-$HT_{2A}$ antagonists mianserin and ketanserin was not affected by $mGlu_2$ co-expression (Fig. 3B, Table 2).

As hallucinogenic 5-$HT_{2A}$ receptor agonists are able to produce $G_{\alpha i}$-dependent signaling responses in addition to $G_{\alpha q}$-mediated signaling pathways (Gonzalez-Maeso et al., 2007) the effect of the hallucinogens DOI, mescaline, and LSD on intracellular cAMP levels

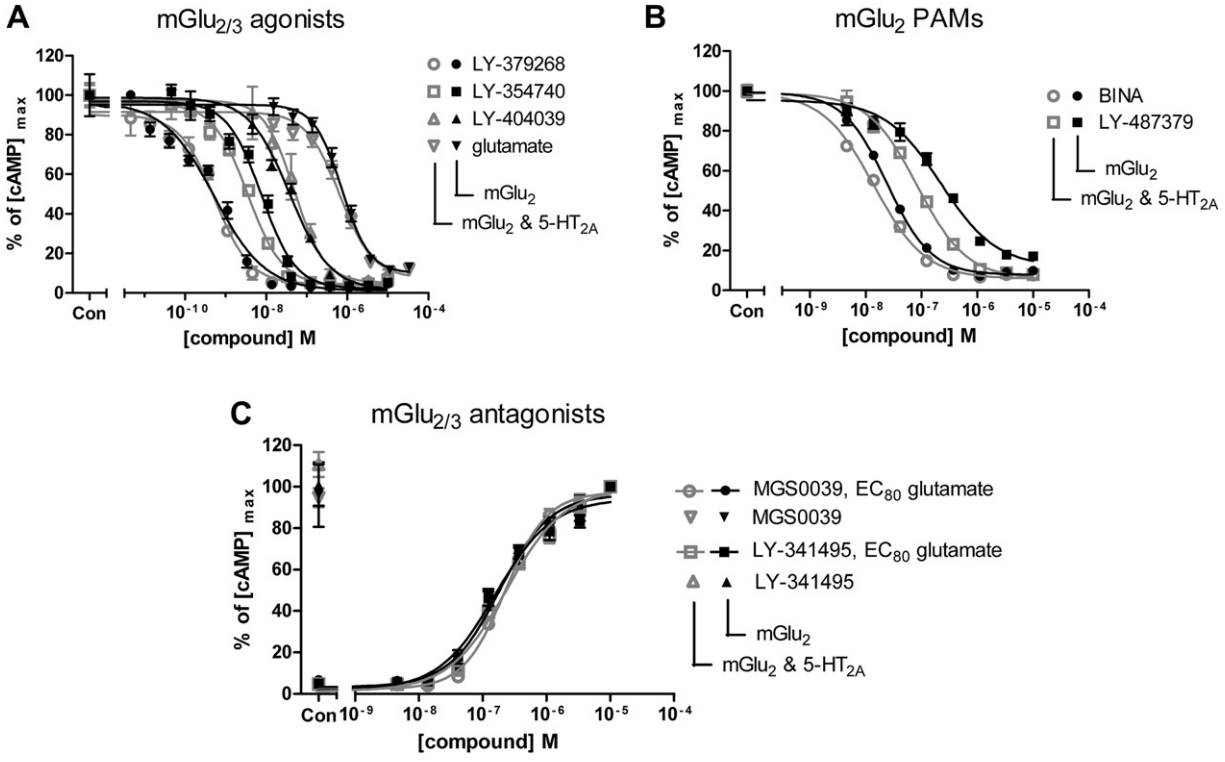


**Fig. 2.** Co-expression of the 5-$HT_{2A}$ receptor results in slightly higher potency of $mGlu_2$ PAMs but does not influence the pharmacology of $mGlu_2$ agonists or antagonists. HEK-293 T-REx solely expressing $mGlu_2$ or co-expressing $mGlu_2$ and 5-$HT_{2A}$ were incubated with different concentrations of $mGlu_{2/3}$ agonists (A), $mGlu_2$ PAMs (B), or $mGlu_{2/3}$ antagonists (C) and inhibition of forskolin-induced cAMP formation was measured. Values are expressed as % of maximal cAMP inhibition. (A) Concentration–response-curves resulting from $mGlu_2$ receptor stimulation are identical among cell lines independent from co-expression of 5-$HT_{2A}$. (B) The $mGlu_2$ PAMs BINA and LY487379 were tested under presence of glutamate $EC_{20}$. Co-expression of 5-$HT_{2A}$ resulted in a slight left shift of the concentration–response-curves. (C) The $mGlu_2$ antagonists MGS0039 an LY341495 were tested under presence of glutamate $EC_{80}$. No difference was observed upon co-expression of the 5-$HT_{2A}$ receptor.

**Table 1**
Potencies of mGlu2 ligands.

| | $mGlu_2$ | $mGlu_2$ and 5-$HT_{2A}$ |
|---|---|---|
| $mGlu_{2/3}$ agonists ($EC_{50}$ in nM) | | |
| Glutamate | 730 (649–820) | 650 (562–752) |
| LY379268 | 0.52 (0.45–0.6) | 0.54 (0.47–0.62) |
| LY354740 | 7.6 (6.8–8.5) | 3.5 (3.2–3.9)[a] |
| LY404039 | 37.9 (32.6–43.9) | 50.1 (40–62.7) |
| $mGlu_{2/3}$ antagonists ($IC_{50}$ in nM) | | |
| MGS0039 | 182 (157–212) | 225 (208–244) |
| LY341495 | 157 (135–183) | 237 (206–273) |
| $mGlu_2$ PAMs ($EC_{50}$ in nM) | | |
| BINA | 24.1 (22.8–25.4) | 14.1 (13.1–15.1)[a] |
| LY487379 | 222 (190–259) | 83.7 (76.6–91.5)[a] |

$mGlu_{2/3}$ agonists, antagonist and PAM potencies were determined in an endpoint cAMP assay in HEK-293 T-REx cells only expressing $mGlu_2$ or co-expressing $mGlu_2$ and 5-$HT_{2A}$. Mean $EC_{50}$ and $IC_{50}$ values are presented in nM, range of the standard deviation is given in parentheses. Antagonist and PAM potencies were measured after co-stimulation with $EC_{80}$ or $EC_{20}$ of glutamate, respectively.

[a] Significant statistical difference $p \leq 0.01$; $n = 6$ (unpaired two-tailed $t$-test).

was measured in 293-$H_{2A}$ and 293-$H_{2A}$-i$G_2$ cells. We could not detect a reduction of forskolin-induced cAMP levels in either cell and with neither of the hallucinogenic 5-$HT_{2A}$ agonists (data not shown). To test if the hallucinogenic $G_{\alpha i}$-mediated signaling was dependent on the activation state of the $mGlu_2$ receptor we pre-incubated the cells with an $mGlu_2$ receptor agonist (LY404039) or antagonist (LY341495) for five hours. However, a cAMP reduction by the hallucinogenic compounds was still not observed (data not shown).

In summary, these results do not support a direct functional interplay of the two receptors when co-expressed in the same cell. In our system, the existence of 5-$HT_{2A}$-$mGlu_2$ heterocomplexes itself does not alter the pharmacology of the receptors. However, a minor modulating effect might still exist, since the potency of two tested $mGlu_2$ PAMs and the agonist LY354740 appeared to be slightly increased in co-expressing cells.

### 3.3. DOI does not affect $mGlu_{2/3}$ agonist binding affinities

We intended to gain further insights into a functional crosstalk between 5-$HT_{2A}$ and $mGlu_2$ in a native environment. The influence of the hallucinogenic 5-$HT_{2A}$ agonist DOI on the affinity of the $mGlu_{2/3}$ agonist LY354740 was assessed in rat cerebral cortex membranes. Gonzalez-Maeso et al. (2008) reported previously that the affinities of $mGlu_{2/3}$ agonists (LY379268, DCG-IV, and L-CCG-I) measured in mouse sensory cortex membranes were lower in the presence of DOI. We measured the displacement of the radioligand [$^3$H]LY341495 by the $mGlu_{2/3}$ agonist LY354740 in the presence or absence of DOI. [$^3$H]LY341495 binding was inhibited by LY354740 in a concentration dependent manner. The data were best fit by a two site model with $K_i$ values of 3.86 nM and 50 nM, reflecting high and low affinity binding of the agonist LY354740. Inclusion of fixed 10 μM DOI to LY354740 concentrations did neither significantly change the shape of the displacement curve nor the resulting $K_i$ values ($K_i = 3.06$ nM and 52 nM) (Fig. 4). Thus, we could not detect a pharmacological interplay of a 5-$HT_{2A}$-$mGlu_2$ heteromer in native tissue.

## 4. Discussion

Our data demonstrate that $mGlu_2$ is indeed found in close proximity to 5-$HT_{2A}$, which was shown using a HTRF-based assay. Furthermore, a potential interaction of $mGlu_2$ with 5-$HT_{2B}$ as well as $mGlu_5$ but not 5-$HT_{2C}$ was observed. However, we could not

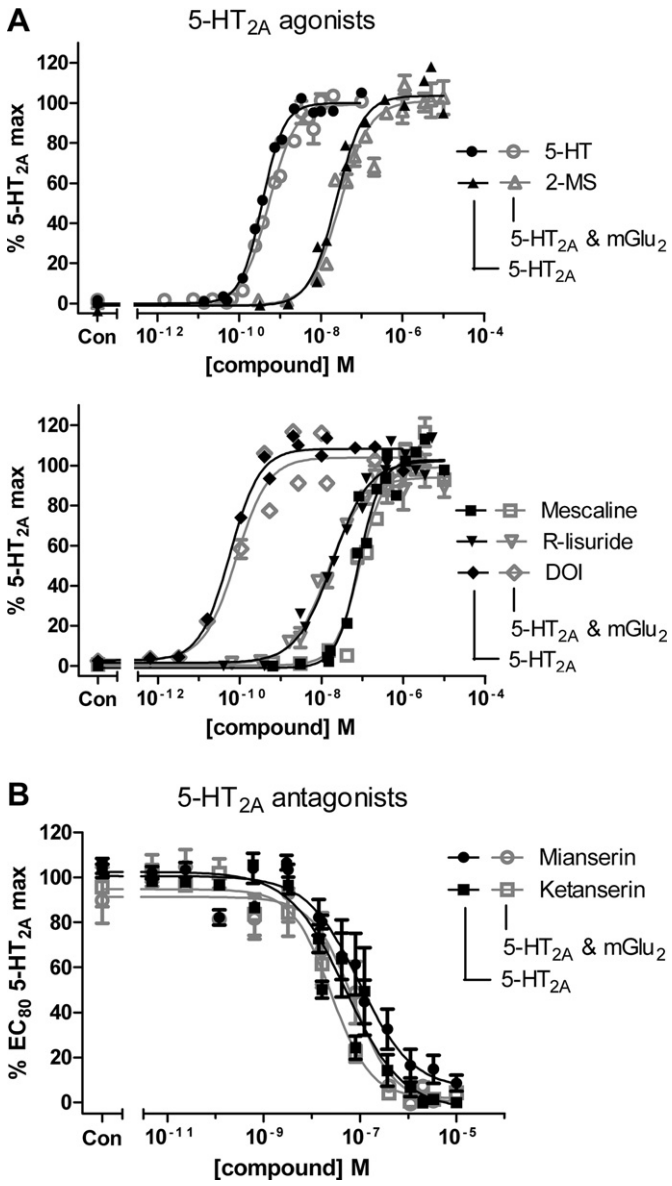


**Fig. 3.** Co-expression of the $mGlu_2$ receptor has no effect on 5-$HT_{2A}$ agonist and antagonist pharmacology. The effect of non-hallucinogenic and hallucinogenic 5-HT agonists (A) and antagonists (B) on $Ca^{2+}$ mobilization in HEK-293 T-REx cells solely expressing the 5-$HT_{2A}$ receptor or HEK-293 T-REx cells co-expressing 5-$HT_{2A}$ and $mGlu_2$ was measured using a FLIPR system. Data were normalized to the maximal fluorescence signal $RFU_{max}$ obtained with 5-HT. Antagonists (B) were tested under presence of 5-HT $EC_{80}$. No differences were observed upon co-expression of $mGlu_2$.

detect a relevant functional crosstalk between 5-$HT_{2A}$ and $mGlu_2$. We tested the potential influence of the 5-$HT_{2A}$-$mGlu_2$ heteromer formation in different functional assays. Potencies and efficacies of $mGlu_{2/3}$ agonists (LY379268, LY354740, and LY404039) and antagonists (LY341495 and MGS0039) were not relevantly affected by the presence of 5-$HT_{2A}$ and only a slight increase of the potencies of the $mGlu_2$ PAMs LY487379 and BINA was observed. Likewise, hallucinogenic (DOI and mescaline) or non-hallucinogenic (5-HT, 2-MS, and R-(+)lisuride) 5-$HT_{2A}$ agonist or antagonist (ketanserin and mianserin) signaling was not changed upon $mGlu_2$ co-expression. In membranes of the mPFC we did not find an influence of DOI on the competition of [$^3$H]-LY341495 binding by LY354740. Furthermore, we did not observe a reduction of cAMP after stimulation with hallucinogenic 5-$HT_{2A}$ agonists.

**Table 2**
Potencies of 5-$HT_{2A}$ ligands.

| | 5-$HT_{2A}$ | $mGlu_2$ and 5-$HT_{2A}$ |
|---|---|---|
| 5-HT agonists, non-hallucinogenic ($EC_{50}$ in nM) | | |
| 5-HT | 0.28 (0.25–0.31) | 0.40 (0.37–0.43) |
| 2-MS | 22.6 (19.6–26.1) | 30.1 (26.8–33.7) |
| R-(+)lisuride | 19.2 (16.3–22.7) | 16.0 (13.7–18.8) |
| 5-HT agonists, hallucinogenic ($EC_{50}$ in nM) | | |
| DOI | 0.05 (0.04–0.06) | 0.07 (0.06–0.08) |
| Mescaline | 84.0 (74.8–94.2) | 85.2 (78.9–92.2) |
| 5-HT antagonists ($IC_{50}$ in nM) | | |
| Ketanserin | 33.3 (26.7–41.4) | 26 (21.3–31.8) |
| Mianserin | 81.1 (59–111.4) | 64.7 (47.4–88.3) |

5-$HT_{2A}$ agonist and antagonist potencies for intracellular $Ca^{2+}$ mobilization were determined in HEK-293 T-REx cells solely expressing 5-$HT_{2A}$ or co-expressing $mGlu_2$ and 5-$HT_{2A}$. Antagonist potencies were measured after co-stimulation with $EC_{80}$ of 5-HT. Mean $EC_{50}$ and $IC_{50}$ values are presented in nM, range of the standard deviation is given in parentheses.

Several studies demonstrated a negative reciprocal influence of 5-$HT_{2A}$ and $mGlu_2$ *in vivo*. Orthosteric $mGlu_{2/3}$ agonists inhibited 5-$HT_{2A}$-induced EPSCs in layer V pyramidal cells in mPFC (Marek et al., 2000) and treatment of mice with the $mGlu_{2/3}$ agonist LY379268 or the $mGlu_2$ enhancer LY566332 attenuated stimulation of PI hydrolysis in the frontal cortex induced by 5-$HT_{2A}$ activation with DOI (Gonzalez-Maeso et al., 2008; Molinaro et al., 2009). Furthermore, administration of the $mGlu_{2/3}$ agonist LY354740 suppressed head shakes induced by 5-$HT_{2A}$ activation with the hallucinogen DOI in mice (Gewirtz and Marek, 2000). In contrast, the $mGlu_{2/3}$ antagonist LY341495 increased EPSCs (Marek et al., 2000) and DOI-induced head shakes (Gewirtz and Marek, 2000). These data are in agreement with the idea that glutamate release in the mPFC is induced by 5-$HT_{2A}$ activation and that $mGlu_{2/3}$ receptors function as inhibitory autoreceptors on glutamatergic neurons.

Several studies have explored the potential co-localization of $mGlu_2$ and 5-$HT_{2A}$ in the cortex, which could explain this functional receptor interaction due to direct receptor multimerization in the same cells. This direct interaction has however not unequivocally been demonstrated so far. Employing radiolabeled ligands for $mGlu_{2/3}$ and 5-$HT_{2A}$ it was shown that [$^3$H]LY354740 binding shows a more diffuse distribution than it is the case with [$^{125}$I]DOI, although there is a similar laminar pattern in the mPFC (Marek et al., 2000). While through binding studies a co-expression of both receptors is predominantly found in layers I and Va of the cortex, with $mGlu_2$ receptors likely localized on thalamocortical afferents, this general pattern does not prove that the two receptor types are indeed expressed in the same cells in the brain (Marek et al., 2001). Fluorescence *in situ* hybridization experiments suggested that $mGlu_2$ and 5-$HT_{2A}$ mRNAs are present in the same cells in layer V mouse somatosensory cortex neurons and in cortical primary cultures (Gonzalez-Maeso et al., 2008), which is a prerequisite for a physiological 5-$HT_{2A}$-$mGlu_2$ heterocomplex. However, a co-localization of the two receptors in neurons of the intact brain investigated at the protein level has not yet been unequivocally demonstrated. Only in a very recent study in cortical slices and neuronal primary cultures the two receptors were found to be co-localized (Fribourg et al., 2011). These results might need to be reproduced to come to a final conclusion on this matter.

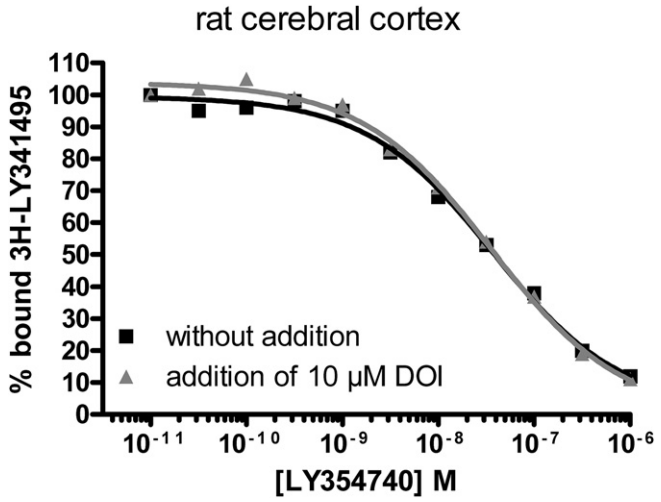


**Fig. 4.** DOI does not affect $mGlu_{2/3}$ agonist competition in rat cortex membranes. [$^3$H] LY341495 displacement curves in rat cerebral cortex membranes. The 5-$HT_{2A}$ agonist DOI (10 µM) did not significantly affect the affinity of the $mGlu_{2/3}$ agonists LY354740 for the glutamatereceptor binding site in rat cerebral cortex labeled with 1 nM [$^3$H] LY341495.

Lately, a number of studies indicate that GPCRs not only function as monomeric units but also as receptor homo- or heteromers. While some GPCRs form obligate homodimers, like mGlu receptors (Brock et al., 2007) or heteromeric receptors among receptors of the same family, like the $GABA_B$ receptor (Maurel et al., 2008), a receptor heteromer may also be formed by divergent GPCRs. Receptor heteromer is defined as a 'macromolecular complex composed of at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components' (Ferre et al., 2009; Pin et al., 2007). Gonzalez-Maeso et al. (2008) reported the existence of a 5-$HT_{2A}$-$mGlu_2$ heterocomplex by resonance energy transfer methods in recombinant cell systems and co-immunoprecipitation from human brain lysates, indicating that the heterocomplex might be responsible for the functional interaction of the two receptor systems. In contrast, $mGlu_2$ did not interact with 5-$HT_{2C}$. We could confirm these data in the present study. We performed careful studies employing a Tag-lite HTRF assay using Clip- and Snap-tagged receptors, which eliminates background and 'bleed-through' signals compared to classical FRET experiments. The Tag-lite substrates are not membrane permeable and only receptor pairs present at the cell surface are labeled and detected (Maurel et al., 2008). We could confirm the close proximity of $mGlu_2$ and 5-$HT_{2A}$ and not of 5-$HT_{2C}$. However, we also observed resonance transfer when we tested if $mGlu_2$ could be able to interact with the 5-$HT_{2B}$ serotonin receptor isoform. Furthermore, the HTRF data indicated an interaction of $mGlu_2$ with $mGlu_5$. This data question the specificity of the 5-$HT_{2A}$-$mGlu_2$ interaction, since (at least) $mGlu_2$ appears to be rather promiscuous. Furthermore, it should be noted that resonance transfer data are no proof or disproof for *direct* protein interactions. However, HTRF methods are less sensitive to correct dipole formation than classical FRET methods (Selvin, 2002), which improves the probability that absence of a signal is indeed due to a longer distance of the receptors.

Heteromer formation between different mGlu receptor subtypes has been observed before (Doumazane et al., 2010), although an $mGlu_2$-$mGlu_5$ heteromer has not been described. The observation of a direct interaction between $mGlu_2$ and $mGlu_5$ is interesting in light of $mGlu_5$ agonists increasing the frequency of EPSCs in the rat mPFC (Marek and Zhang, 2008). Glutamate release in the mPFC is modulated by several other receptor types in addition to 5-$HT_{2A}$ and $mGlu_2$. Thus, whether for example $\alpha_1$-adrenergic and orexin$_2$ receptors, which stimulate glutamate release, can substitute for 5-$HT_{2A}$ receptors in heteromers and $mGlu_4$, $mGlu_8$, µ-opioid, and adenosine $A_1$ receptors, which suppress glutamate release, can substitute for $mGlu_2$ receptors would be an additional test of the 5-$HT_{2A}$-$mGlu_2$ heteromer hypothesis (Marek and Zhang, 2008). In addition, the 5-$HT_{2A}$ receptor is capable to heteromerize with the dopamine $D_2$ receptor and this heterocomplex formation

results in functional receptor crosstalk (Albizu et al., 2011). These data suggest that expression of two receptor types in one cell line often leads to formation of heterocomplexes among those receptors.

These observations raised the question whether heteromer formation of 5-$HT_{2A}$ and $mGlu_2$ is indeed responsible for the functional crosstalk of the receptors in the intact brain. Given that two receptors are expressed in the same cell, crosstalk can result from different kinds of interactions: a) from heteromerization, b) from functional crosstalk of the intracellular signaling without heteromerization of the receptors, or c) from functional crosstalk of the intracellular signaling although the receptors form heteromers (Prezeau et al., 2010). Such oligomerization-independent signal integration has for example been described for receptor pairs like $GABA_B$ and $mGlu_{1A}$ or $CB_1$ and μOR (Canals and Milligan, 2008; Rives et al., 2009).

To investigate the functional relevance of the 5-$HT_{2A}$-$mGlu_2$ heterocomplex, we addressed the intracellular signaling in stable cell lines expressing either one or both receptors. The pharmacology of $mGlu_2$ ligands was not affected by the presence or absence of 5-$HT_{2A}$. Thus, we could not confirm a functional consequence of the heteromer formation for $mGlu_2$ signaling, as has been shown in a very recent study by Fribourg et al. (2011). There an increase of $G_{\alpha i}$ and decrease of $G_{\alpha q}$ signaling of the heteromeric 5-$HT_{2A}$-$mGlu_2$ receptor compared to the respective homomeric receptors was reported by measuring channel activities in *Xenopus* oocytes. In a previous study the same group reported a decrease of the high affinity activation of $G_{\alpha q/11}$ by the 5-$HT_{2A}$ agonist DOI in the presence of $mGlu_2$ measuring [$^{35}$S]GTP-γS bound to $G_{\alpha q/11}$, which was reversed by the $mGlu_2$ agonist LY379268 (Gonzalez-Maeso et al., 2008). In mouse cortical neurons the inverse $mGlu_2$ agonist LY341495 enhanced the $G_{\alpha q}$ signaling ($Ca^{2+}$ release) evoked by 5-HT (Fribourg et al., 2011). We addressed the $G_{\alpha q/11}$ activation of 5-$HT_{2A}$ by determination of the intracellular $Ca^{2+}$ mobilization. In our cells, 5-$HT_{2A}$ signaling was not influenced by co-expression of $mGlu_2$. This was observed for hallucinogenic and non-hallucinogenic compounds. We explored the pharmacological modulation of receptor-specific compounds based on receptor co-expression while the study of Fribourg et al. (2011) focused their attention more to the relevance of the receptor system for signaling of antipsychotic drugs with a limited number of compounds. Besides different assay systems, receptor expression levels, or sensitivities of the read outs also the different approach might be an explanation for the discrepancies of the data. Exploration in future studies including co-stimulation of the receptors, which was described to result in a shift in receptor signaling, and antipsychotics like clozapine will help to reveal the meaning of these shifts in the mammalian systems.

Besides the 'regular' $G_{\alpha q/11}$-mediated signaling, hallucinogens have previously been demonstrated to induce also $G_{\alpha i}$ activation through 5-$HT_{2A}$ stimulation. This can lead to the induction of certain hallucinogen-specific genes like egr-2 (Gonzalez-Maeso et al., 2007). $G_{\alpha i}$ signaling of 5-$HT_{2A}$ has been shown to be increased by $mGlu_2$ co-expression but decreased in presence of a $mGlu_2$ agonist (Gonzalez-Maeso et al., 2008). On the other hand was the inverse 5-$HT_{2A}$ agonist clozapine reported to increase $mGlu_{2/3}$ agonist DCG-IV-induced $G_{\alpha i}$ signaling in mouse cortical membranes in a 5-$HT_{2A}$-dependent manner (Fribourg et al., 2011). We intended to address the hallucinogenic $G_{\alpha i}$-mediated 5-$HT_{2A}$ signaling by cAMP measurement, but could not observe an effect of the hallucinogenic compounds at any condition tested. If $G_{\alpha i}$ proteins are activated by hallucinogenic 5-$HT_{2A}$ ligands this does at least in our cell system not translate into inhibition of the adenylate cyclase. However, we cannot exclude that certain proteins required for hallucinogen-specific signaling (and receptor crosstalk) might be missing in our cells. But our data indicate that the differences between hallucinogenic and non-hallucinogenic 5-$HT_{2A}$ activation is more complex and may not be based on simple second messenger systems. Although differential gene activation may be a possibility (Gonzalez-Maeso et al., 2007, 2003), this cannot explain immediate behavioral differences as they are for example observed in the mouse head twitch model. Complex interactions involving further receptors or second messengers could play a role (Schmid and Bohn, 2010).

Allosteric interactions between the protomers of a protein complex can result in differential ligand binding properties of the integrated receptors. This has been reported for 5-$HT_{2A}$ and $mGlu_2$ using mouse sensory cortex (Gonzalez-Maeso et al., 2008; Moreno et al., 2011). We did not observe an effect of DOI on $mGlu_{2/3}$ agonist binding in rat cerebral cortex membranes. Thus, our data do not support a direct allosteric interaction of $mGlu_2$ and 5-$HT_{2A}$. However, data from $mGlu_2$-knock out mice indicated that $mGlu_2$ is necessary for high-affinity binding of DOI and hallucinogen-specific 5-$HT_{2A}$ responses like egr-2 induction and head twitch behavior (Moreno et al., 2011). These discrepancies might be due to the different species and tissues used or based on the well-known cross-talk of the two receptor systems in the intact brain.

In summary, our data do not put into question the functional interaction of the two receptor systems and do support a direct interaction of $mGlu_2$ and 5-$HT_{2A}$ in recombinant cell systems. However, we did not find evidence for a functional crosstalk of the signaling cascades induced by the receptor heteromer itself, suggesting that the signal integration does not occur at the receptor level. Furthermore, the 5-$HT_{2A}$-$mGlu_2$ interaction does not appear to be selective, indicated by the positive HTRF signals we obtained for example for $mGlu_2$ and 5-$HT_{2B}$. This observation might, however, be due to the overexpression system. But receptor interactions must not necessarily have a functional consequence. Further studies are required to clarify if the 5-$HT_{2A}$-$mGlu_2$ heteromers indeed exist *in vivo* and if such heteromers have a physiological relevance. If the *in vivo* relevance of such heteromers can be proven, they may play a role in pathological conditions like schizophrenia and would therefore be an interesting target for treatment options.

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## Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.neuropharm.2012.01.010.

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